Vol. 167, No. 1, 1990 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 28, 1990 Pages 143-147

INHIBITION OF LYSOPHOSPHOLIPASE BY CHOLESTEROL IN RABBIT AORTA

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Received December 22, 1989

Lysophospholipase activity was measured in rabbit aorta using 1-[1-^{14}C] palmitoyl-*sn*-glycero-3-phosphocholine as a substrate. The enzyme did not require Ca^{2+} for its activation and the maximal activation was attained in the presence of EGTA. Cholesterol dose-dependently inhibited the lysophospholipase activity in the soluble fraction and IC_{50} value was approximately 15 μM. Lineweaver-Burk plot revealed that cholesterol competitively inhibited lysophospholipase and Km values in the presence and absence of cholesterol (15.5 μM) were 12.3 and 2.8 μM, respectively. Vmax values were approximately 475 pmol/min·mg. The results suggest that cholesterol can interact with the enzyme *per se*, resulting in the inhibition of the lysophospholipase activity in rabbit aorta. © 1990 Academic Press, Inc.

Lysophospholipid, an amphiphilic molecule that can change the properties of membranes and cause various biological effects (1), is generated by phospholipases *in situ* or by lecithin:cholesterol acyl transferase in plasma (2). Arterial tissues contain two enzymatic pathways to control the level of lysophospholipid *in situ* : 1) fatty acyl CoA: lysophosphatide fatty acyl transferase which catalyzes the formation of phospholipid from lysophospholipid and 2) lysophospholipase which hydrolyzes lysophosphatidylcholine (lysoPC) to glycerophosphocholine and fatty acid (3,4). In atherosclerotic aorta, several-fold increase in lysoPC level was demonstrated in nutritionally induced atherosclerosis (5,6). The difference of lysoPC concentrations between atherosclerotic and lesion-free areas might be explained as a result of differing rates of uptake of lysoPC from plasma (5). Alternatively, there might be an impairement of the activities of the enzyme(s) responsible for the removal of this molecule taken up by the tissue. These findings led us to investigate whether changes of catabolism of lysoPC might be present in atherosclerotic aorta, contributing to the increased level of lysoPC. The present study demonstrates that cholesterol competitively inhibits the lysophospholipase activity in rabbit aorta.

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Abbreviations: lysoPC, lysophosphatidylcholine; [^{14}C]lysoPC, 1-[1-^{14}C]palmitoyl-*sn*-glycero-3-phosphocholine; [^{14}C]PC, 1-palmitoyl-2-[1-^{14}C]arachidonoyl-*sn*-glycero-3-phosphocholine.

0006-291X/90 $1.50
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The results may imply the importance of the attenuated lysophospholipase activity as the responsible mechanism(s) for the enhanced accumulation of lysoPC in atherosclerotic vessels.

MATERIALS AND METHODS

Preparations of the soluble and particulate fractions Japanese white rabbits weighing approximately 2.5 kg were killed by an intravenous injection of air. The thoracic aorta (from aortic arch to diaphragm) was excised and placed immediately in homogenizing buffer at 4°C (0.25 M sucrose, 10 mM imidazole, 10 mM KCl, 100 mM NaCl, 1 mM dithiothreitol, 150 μg/ml benzamidine, 100 μg/ml bacitracin, pH 8.0). The aorta was freed of surrounding connective tissue, minced and homogenized in a 12 volume of homogenizing buffer using a hand-driven glass homogenizer (approximately 1 g of tissue/12 ml of buffer). The homogenized tissue was then centrifuged at 600 x g for 10 min to remove nuclei and cellular debris and the resulting supernatant fraction was centrifuged again at 100,000 x g for 60 min to separate the particulate fraction from the soluble fraction. Particulate fraction was resuspended in homogenizing buffer.

Assay of lysophospholipase 1-[1-^{14}C]palmitoyl-*sn*-glycero-3-phosphocholine ([^{14}C]lysoPC, 58.5 mCi/mmol) with or without cholesterol was dried under a stream of N_2 gas and dissolved in ethanol just prior to use (7). Lysophospholipase activity was routinely assayed in a 0.25 ml reaction volume containing 50 mM Tris·Cl (pH 7.4), 10 mM Ca^{2+} or 5 mM EGTA as indicated in the text, and 50 μl of either soluble or particulate fractions. Reaction was initiated by addition of 10 μl of the radiolabelled substrate (finally 8 μM) in the presence or absence of cholesterol and the incubation was carried out at 37°C for 10 min. The reaction products were extracted with 100 μl of n-butanol, the layer was clarified by centrifugation at 200 x g for 5 min, and the reaction products were separated by thin-layer chromatography utilizing Silica G plates and a mobile phase of petroleum ether/diethyl ether/acetic acid (70/30/1). The spots were visualized by a I_2 vapour, scraped and counted. In separate experiments, phospholipase A_2 activity was assayed utilizing 1-palmitoyl-2-[1-^{14}C]arachidonoyl-*sn*-glycero-3-phosphocholine ([^{14}C]PC, 51.7 mCi/mmol) as a substrate. Lysophospholipase and phospholipase A_2 activities were expressed as pmol product formed/min per mg protein.

The radioactive compounds were obtained from New England Nuclear. Other chemicals were purchased from Sigma. Protein concentration was determined with the use of the Bio-Rad protein assay kit with bovine serum albumin as a standard.

RESULTS AND DISCUSSION

Soluble and particulate fractions in rabbit aorta were incubated with [^{14}C] lysoPC or [^{14}C]PC; the hydrolytic activities of [^{14}C]lysoPC and [^{14}C]PC were 642 ± 83 and 38 ± 3 pmol/min·mg in the soluble fraction (n=4), and 507 ± 112 and 39 ± 6 pmol/min·mg in the particulate fraction (n=4). Preferential hydrolysis of [^{14}C]lysoPC indicated that the lysophospholipase activities was distinct from phospholipase A_2. Lysophospholipase activities from different organs from the rabbit were measured and it was found that the activity in aorta was approximately 2 times as high as the activities of any other sources tested (heart, liver and

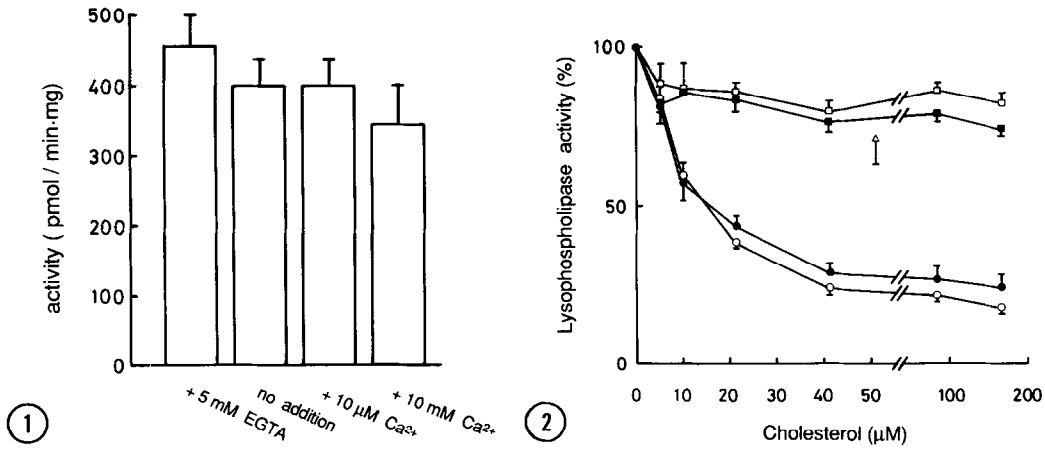

Fig. 1. Ca^{2+} requirement for the optimal lysophospholipase activity in the soluble fraction of rabbit aorta. Standard reaction mixtures in a total volume of 0.25 ml contained 40-50 μg protein, 50 mM Tris·Cl (pH 7.4), 8 μM [^{14}C]lysoPC and 5 mM EGTA or Ca^{2+} as indicated. The data represent the mean ± S.E. of 5 to 6 experiments. Each value did not differ significantly.

Fig. 2. Effects of cholesterol on the lysophospholipase activities in the soluble and particulate fractions. Standard reaction mixtures containing 5 mM EGTA (closed symbols) or 10 mM $CaCl_2$ (open symbols) were incubated with 50 μl of the soluble (●,○) or particulate (■,□) fractions in the presence of the indicated concentrations of cholesterol. In separate experiments, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (55 μM) instead of cholesterol was added to the reaction mixtures containing 5 mM EGTA (△). Data are expressed as % maximal activity and each point is the mean ± S.E. of 3 experiments.

lung)(data not shown). Ca^{2+} requirement of lysophospholipase in the soluble fraction was examined in the presence of 5 mM EGTA or at various concentrations of Ca^{2+} (Fig. 1). The rate of the lysoPC hydrolysis was more rapid, although not significant, in the presence of EGTA than in the presence Ca^{2+} or in the absence of exogenously added Ca^{2+}. The result indicated that lysophospholipase in aorta did not require Ca^{2+} for its activation.

Lysophospholipase activity in the soluble fraction of rabbit aorta was inhibited by cholesterol (Fig. 2). Inhibition was dose-dependent and the half-maximal inhibition was attained at approximately 15 μM (=IC_{50}). Ca^{2+} did not affect the inhibiting effect of cholesterol. In separate experiments, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (PC) was incubated with the assay mixtures and it was found that PC (55 μM) was less potent than cholesterol in inhibiting lysophospholipase. The inhibition by cholesterol was not observed in the range of 5 to 165 μM when the particulate fraction was used as the source of lysophospholipase (Fig. 2), and more than 30 times of cholesterol was necessary to elicit the 50% inhibition (IC_{50}=518 μM) as compared with the IC_{50} value (15 μM) obtained using the soluble fraction. The inhibiting effect induced by cholesterol was further analyzed utilizing Lineweaver-Burk plot. Fig. 3 shows one of the representative tracings and it was revealed that cholesterol competitively inhibited lysophospholipase in the soluble fraction.

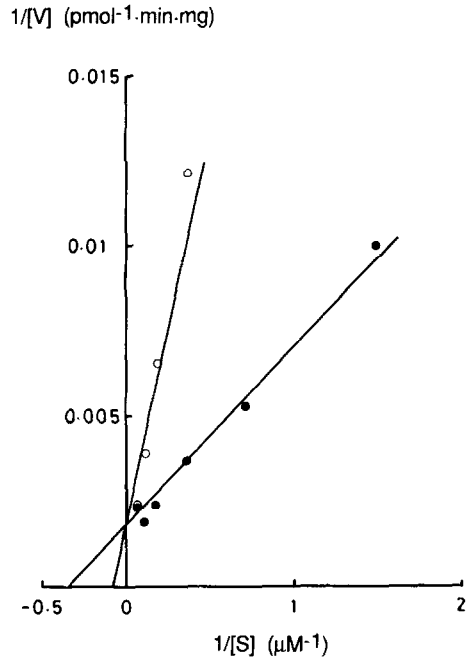

Fig. 3. Lineweaver-Burk plot of the lysophospholipase activity in the presence of EGTA (5 mM). Varying concentrations of [^{14}C]lysoPC were incubated with the soluble fraction in the presence (○) or absence (●) of cholesterol (15.5 μM) for 10 min at 37°C.

Kinetic data showed that the Km values significantly increased in the presence of 15.5 μM cholesterol (from 2.78 ± 0.40 to 12.3 ± 1.25 μM, n=4) without changing the Vmax values (475 ± 48 vs. 476 ± 41 pmol/min·mg).

The inhibiting effect induced by cholesterol was not mediated by the interaction of cholesterol with the radiolabelled lysoPC used as a substrate since 1) the effect of cholesterol was dose-dependent and was more potent than that of equimolar PC, 2) marked difference was noticed for cholesterol concentrations to inhibit lysophospholipses in the soluble and particulate fractions, which eliminated the possibility of the direct interaction of cholesterol with lysoPC since it was speculated that the enzyme in the particulate fraction could be inhibited to the same extent as that in the soluble fraction under such condition, and 3) the kinetic analysis by Lineweaver-Burk plot revealed that cholesterol inhibited lysophospholipase in a competitive manner.

It was found that the concentration of lysoPC in atherosclerotic aorta from squirrel monkeys was nearly eight times higher than that in control lesion-free area (5,6). The authors speculated that the increased rate of uptake of lysoPC from plasma might be responsible for the enhanced accumulation of this molecule in atherosclerotic aorta (5). The present result suggests that accumulation of lysoPC may be further acceralated particularly when catabolism of lysoPC is inhibited in atherosclerosis. Competitive inhibition of lysophospholipase by L-palmitoyl-

carnitine was reported in ischemic myocardium where the enhanced accumulation of lysoPC was also found (7,8,9). LysoPC can affect ionic transport and conductance in a number of systems, including inhibition of sarcolemmal Na^+/K^+ ATPase (10), increase in calcium flux (11,12) and inhibition of sarcolemmal Na^+/K^+ exchange (13). Important is the role of lysoPC as the responsible mechanism(s) for the malignant arrhythmias during myocardial ischemia (14). LysoPC was also reported to alter the muscle tonus in guinea pig ileum and rabbit aorta (15,16). Thus, the abundance of lysoPC may be, in part, be resposible for the altered vascular reactivity in atherosclerosis (17).

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